Imaging techniques: **Picture the world with kaleidoscope dyes**

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Advances in fluorescence light microscopy have facilitated the production of images from multiply labeled specimens.

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Current Biology 1997, 7:R182–R185

Mapping the relative distributions of biologically important molecules to domains within cells and tissues is vital to understanding their functions. A key technique required for these localization studies is fluorescence light microscopy [1], which uses various fluorescent dyes attached to antibodies, DNA, RNA or other molecules to visualize molecules in specific regions of cells. The technique was first used some 50 years ago to look at cells growing in tissue culture [2], and methods for visualizing more than one protein in the same cell were subsequently developed [3]. Preparation of the specimen is perhaps the most important step of the entire imaging process. A typical multiply labeled specimen is prepared using several fluorescently labeled probes [4]; in the case of immunofluorescence, these are affinity purified antibodies raised in different animal species [5], and for fluorescent *in situ* hybridization (FISH), fluorescently labeled DNA or RNA fragments are used [6].

In order to optimize signal collection from multiply labeled specimens, it is necessary to choose fluorochromes which have excitation and emission spectra that do not overlap significantly. This facilitates efficient optical filtering (using multiple bandpass filters) and reduces the chances of the signal from one fluorochrome bleeding through into that of another. Two traditional fluorochromes for these preparations are fluorescein (green emission) and rhodamine (red emission), although a whole new battery of dyes which have excitation and emission maxima that span the useful spectrum for light microscopy have appeared steadily over recent years (Table 1). These dyes can be conjugated to antibodies for immunofluorescence or to a nucleotide, such as deoxyuridine 5′-triphosphate (dUTP) for FISH, and are, of course, readily available from commercial sources. Many of these fluorescent dyes are used in various multicolour applications in addition to fluorescence microscopy, including non-isotopic sequencing [7,8], multicolour flow cytometry [9] and for screening microarrays of DNA [10].

Various staining strategies have been developed for increasing the fluorescent signal above the background 'noise' of the specimen. These amplification techniques include the use of various antibody combinations to boost the signal -— for example, combinations of fluorescein and anti-fluorescein [11]; the development of brighter fluorescent dyes, such as cyanine 3 and tyramide [12]; or the use of dyes with excitation maxima outside the range of tissue autofluorescence, such as cyanine 5 [13]. Moreover, the important problem of resolving fluorescently labeled biological structures within light-scattering tissues has been solved in several ways with the development of confocal microscopy [14], computer methods to calculate and remove out-of-focus fluorescence by deconvolution [15], and two-photon microscopy [16], so that fluorescence microscopy can now be applied to a wider variety of relatively thick samples.

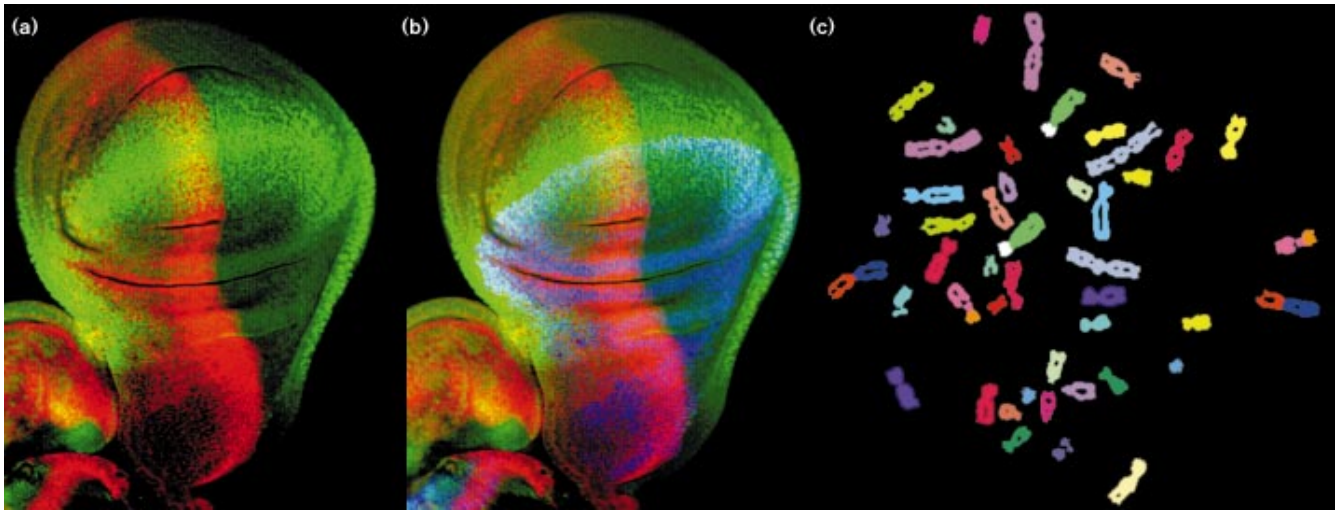
The laser-scanning confocal microscope has been used extensively for visualizing at least three different fluorescent probes simultaneously in thicker tissue samples (up to 200 μm in favourable specimens; Fig. 1a,b). Here, the essential features for combining three separate one-colour images into a single multicolour image are the elimination

Table 1

Peak excitation and emission wavelengths of some commonly used fluorophores and examples of nuclear counterstains.

Dye	Excitation maximum (nm)	Emission maximum (nm)
Coumarin	350	440
Fluorescein isothiocyanate	496	518
Bodipy	503	511
Cyanine 3	554	568
Tetramethylrhodamine	554	576
Lissamine rhodamine	572	590
Cyanine 3.5	581	588
Texas Red	592	610
Cyanine 5	652	672
Cyanine 5.5	682	703
Cyanine 7	755	778
Nuclear dyes		
Hoechst 33342	346	460
4,6-Diamidino-2-phenylindole	359	461
Acridine orange	502	526
Propidium iodide	536	617
TOTO3 (a dimeric cyanine dye)	642	661

Figure 1



(a,b) *Drosophila* third instar wing imaginal disc double-labeled (a) or triple-labeled (b) for expression of wing-patterning genes. The Vestigial protein is shown in green, the Apterous protein in blue, with a region of Apterous staining that bisects the vestigial domain in light blue, and the CiD protein that marks cells of anterior fate in red (see [20] for more details). (c) An example of a spectral karyotype of human chromosomes using classification colours (see [27]).

of background fluorescence and the ability to collect the images from precisely the same region of the specimen [17]. Various methods of multicolour confocal imaging have been described [18], and modern confocal microscopes equipped with three sensitive photomultiplier tube detectors are able to collect three images simultaneously at three different excitation wavelengths. Three-colour confocal imaging can be achieved with a single krypton/argon laser light source, which produces three major peaks at 488 nm (blue), 568 nm (yellow) and 647 nm (red). These wavelengths correlate well with the excitation spectra of some commonly used fluorochromes including fluorescein, rhodamine and cyanine 5 (Table 1). Yet more fluorochromes can be imaged by adding more lasers and filters to the confocal system, though this can prove to be expensive.

Multi-label confocal microscopy has been used by developmental biologists in cell lineage studies [19], and in studies of pattern formation, by imaging the relative distribution of three different gene products within a single specimen [20]. More recently, multicolour confocal analysis has become popular for looking at *Drosophila* tissues prepared using various genetic methods [21]. For example, a clone of cells that expresses a specific gene product can be induced to develop in an ectopic position within a developing field of cells. The temporal and spatial effects of the ectopically expressed gene product are subsequently visualized using multicolour confocal microscopy, which detects the clone of cells that is usually marked with a fluorescently labeled Myc-epitope tag, as well as revealing other landmarks of interest within the tissue.

Using the '*flp*-out' technique, in which the *flp*-encoded recombinase is expressed in particular cells to alter their gene expression, Basler and Struhl [22] were able to visualize, by two-colour confocal microscopy, the Hedgehog protein in ectopic positions in *Drosophila* leg and wing imaginal discs. The adult phenotype of a duplicated leg or wing was traced to the effects of ectopically expressing *hedgehog* in the imaginal discs. The *flp*-out technique has also been used to probe the action of Decapentaplegic in *Drosophila* wing tissues [23]. Recently, the Hedgehog protein was ectopically expressed in the developing brains of *eyeless* mutant flies using *flp*-out clones [24], and in this study, which used triple-label confocal microscopy, neuronal differentiation was observed to be confined to those regions of the brain where the Hedgehog protein was ectopically produced.

Recent advancements in multicolour fluorescence microscopy border on the revolutionary, and have been developed for the simultaneous imaging of over 20 different probes within the same specimen — for example, for imaging all 23 human chromosomes using FISH or 'chromosome painting'. In this type of study, rather than using antibodies raised in many different animal species, different fragments of fluorescently labeled DNA that bind to specific sites along chromosomes are used — single fragments in the FISH technique and multiple fragments in the chromosome painting technique. The different DNA probes are labeled with five dyes, both singly and in various combinations with each other. This literally imparts each DNA probe with its own specific 'spectral signature', which can then be detected using fluorescence microscopy.

As most chromosome preparations are extremely flat, they are usually imaged using conventional epifluorescence microscopy with a charge-coupled device camera as the detector. Images of intact nuclei can be improved using deconvolution [25], and perhaps also using a recently described real-time confocal microscope that uses a white light source rather than a laser [26]. The major challenges that had to be overcome in imaging a specimen labeled with 20 or more fluorescent probes were the detection of the spectral signature of each probe using multiple band-pass filters, and the development of computer programs that could analyze and display a merged image of the 20 or so probes in different colours. Two approaches have recently been reported for analyzing such chromosome preparations [27,28].

The first method [27] collects single images at the various excitation wavelengths of the five individual dyes, and then combines all of the images, using computer software to align each image to a counter-stained image of all of the chromosomes (for examples of counterstains, see Table 1). The software also quantitates the intensity of each fluor and uses a colour 'look-up table' to assign each chromosome with a pseudocolor that is based upon its computed grey value and coded to its specific DNA label (Fig. 1c). The second method [28] collects and measures the full-colour spectrum of the entire image, pixel by pixel, and a computer program assigns red, green and blue values to the images based upon the measured spectra from the DNA probes. The spectra are further analysed by Fourier transformation, and different classification colours are assigned to the images. This technique is extremely sensitive and can distinguish between chromosomes labeled with cyanine 3 (emission maximum 568nm) and Texas Red (emission maximum 610nm).

These 'spectral karyotyping' methods are proving to be rapid and accurate approaches to chromosomal mapping and gene localization, and promise to be invaluable for routine clinical testing for chromosomal defects in various genetic disorders, including Down's syndrome and some cancers, such as leukemia. Spectral karyotyping has also been applied to the analysis of evolutionary relationships between species by comparative cytogenetics [28]. In studies of this type, the chromosomes of one species are 'painted' with probes from a closely related species, and, after a single round of hybridization, are analyzed using spectral karyotyping.

All of these recent advances in multicolour imaging continue to provide insights into the organization, development and pathology of cells. The number of applications for fluorescence microscopy continues to increase with the introduction of brighter and more efficient fluorescent dyes, improved methods of specimen preparation, the development of more sophisticated computer imaging algorithms, and the incorporation of new technologies — such as improved laser light sources and faster computers — into the fluorescence microscopes themselves. Perhaps the next challenge is to collect multicolour images from living specimens; for example, living *Drosophila* embryos have been successfully studied using double-label confocal microscopy [29]. The continued improvement of deconvolution techniques, the introduction of two-photon microscopy and the development of improved vital dyes, such as spectral variants of green fluorescent protein [30], promises much for multicolour live-cell analysis.

Acknowledgements

I thank Jim Williams for the *Drosophila* wing imaginal disc specimen (Figure 1a,b), Nadean Brown for suggestions concerning the manuscript and Sean Carroll for our continued work together.

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